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Neurobiological Responses to Stereotactic Focal Irradiation of the Adult Rodent Hippocampus

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Abstract

Radiation effectively treats brain tumors and other pathologies but dose and treatment plans are limited by normal tissue injury, a major cause of morbidity in survivors. Clinically significant normal tissue injury can occur even with therapies that target pathological tissue and limit out-of-target irradiation. Elucidating the mechanisms underlying normal tissue injury is facilitated by studying the effects of focal irradiation and comparing irradiated and un-irradiated tissue in experimental animals. Young adult rats were irradiated using the Leksell Gamma Knife[®] with a 10 Gy maximum dose directed at the left hippocampus and shaped to minimize irradiation contralaterally. At least 95% of targeted hippocampus received ≥ 3 Gy, while all points in the contralateral hippocampus received < 0.3 Gy. Neuronal and microglial markers of damage were assessed in the targeted and contralateral hemispheres of Gamma Knife[®]-treated rats and compared to non-irradiated controls. Acute cell death and sustained changes in neurogenesis and in microglia occurred in the dentate gyrus of the targeted, but not the contralateral, hippocampus, providing experimental evidence that focal irradiation at doses received by peri-target regions during targeted radiation therapy produces robust normal tissue responses. Additional studies using this approach will facilitate assessment of in vivo dose responses and the cellular and molecular mechanisms of radiation-induced brain injury.

Keywords

radiation injury; stereotactic radiosurgery; inflammation; microglia; neurogenesis; Gamma Knife[®]

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1. Introduction

Radiation therapy (RT), in the form of whole brain irradiation (WBI), partial brain irradiation, and stereotactic radiosurgery (SRS), is integral in the management of brain pathologies, particularly neoplasms. The goal of radiation treatment planning is to optimize radiation dose delivered to tumor(s) while minimizing the dose and volume of irradiated normal brain tissue, in order to reduce the likelihood and severity of toxicities.

Contemporary modalities of intensity modulated radiation therapy (IMRT) [1], [2], [3] [4] and SRS [5] [6] were developed to provide relative sparing of normal brain tissue. For example, the Leksell Gamma Knife® (GK) uses image-guided stereotactic coordinates and 201 focused γ -ray sources to target ionizing radiation to intracranial targets. A steep dose gradient at the field edge enables a high dose to be delivered to a target while significantly limiting the dose to surrounding tissue. Late toxicities of targeted forms of RT are reduced relative to WBI, but impairment, including deficits in domains of cognitive function, still may occur [7] [8] [9].

The mechanisms of radiation-induced brain injury and cognitive changes remain unclear. The breadth of dysfunction that can occur, the correlation with dose and volume irradiated, and a variety of direct experimental evidence suggest neural deficits involve changes in the initially healthy brain tissue outside of the region(s) targeted for high-dose irradiation. The threshold, dose-response, and mechanisms of normal tissue injury remain incompletely understood. Normal tissue may be affected by inflammatory and oxidative stress processes associated with necrosis within the targeted tumor(s). In addition, tissue surrounding the targeted tumor(s) may undergo direct, radiation-induced changes since, even with the dose gradients provided by clinical SRS methods, tissue surrounding a target receives a dose that is in a range that produces neural changes in well-studied animal models. A single dose of WBI at 0.5 to 10 Gy in adult rodents induces acute apoptosis of neural progenitor cells and chronically reduces neurogenesis in the hippocampal dentate gyrus (DG) and in the subventricular zone [10] [11] and may cause cognitive impairment [12] [13] [14] that is associated with a sustained inflammatory response [15] [16]. Finally, at least in principle, neural changes could occur in more distant tissue that receives a very low dose (< 0.5 Gy) or is not irradiated at all, as a result of radiation-induced changes in intercellular signaling that may propagate throughout the brain.

Comparing irradiated and unirradiated tissue in individual animals facilitates analysis of the mechanisms of brain injury that follows SRS and other types of brain RT and development of new treatment strategies to ameliorate neural damage. Most experimental animal studies of the neurobiological effects of brain RT have used a single dose of WBI from 0.5 to 10 Gy (see above) or a larger total dose of fractionated WBI [17] [18] [19] [20] [21] [22] [23]. These experiments have greatly expanded the current understanding of the mechanisms of radiation-induced normal tissue injury. How normal brain tissue responds to focally restricted irradiation, as occurs within and around tumor regions during targeted RT, and whether/how unirradiated or minimally irradiated tissue responds to radiation that is targeted to adjacent or distant areas, are less clear. Several laboratories have reported using the GK device for focal irradiation of the rat brain, targeting one hemisphere and comparing cellular and molecular changes in the targeted versus the “un-irradiated” hemisphere [24] [25] [26] [27], and/or reporting changes outside the targeted region [28] [29]. Significantly, however, even with the relatively steep gradient provided by the GK, the high target doses (60–100 Gy) used in these studies expose most or all of the brain to several Gy; they are similar to WBI with a high dose SRS “boost” to a target region.

In the current study, we developed and validated an adult rodent model that permitted assessment of neurobiological response(s) to focal irradiation targeted to a single

hemisphere. We modeled irradiation levels in the target hemisphere to the peri-target dose in clinical SRS and, importantly, tested for potential treatment-induced responses in tissue outside the target that received very little or no irradiation. Using the GK, a 10 Gy maximum dose was targeted to the left dorsal hippocampus of young adult rats and was shaped to minimize irradiation of the contralateral hippocampus (Figure 1). Sensitive markers of acute and longer-term radiation-induced and inflammatory-mediated cellular changes were analyzed in the DG in the irradiated hippocampus and compared to the non-targeted, contralateral hippocampus and to the hippocampus of sham irradiated, age-matched control animals.

2. Materials and methods

2.1 Animals

The animal facility at the Wake Forest University School of Medicine is accredited by the American Association for Accreditation of Laboratory Animal Care and complies with all Public Health Service-National Institutes of Health and institutional policies and standards for laboratory animal care. The Institutional Animal Care and Use Committee approved all protocols. Seven-month-old male Fischer 344 × Brown Norway (F344×BN) F₁ hybrid rats (Harlan Laboratories, Inc.) were acclimated for 4 weeks before use, pair housed (one irradiated and one sham irradiated), and weighed weekly to monitor general health; there was no significant difference in average body weight between irradiated and sham irradiated rats at any time. In Group 1, five rats (three irradiated and two sham irradiated) were treated at 8 months of age and euthanized 6 hours later. In Group 2, thirty rats were analyzed at 1, 7, or 70 days following GK or sham irradiation at 8 months of age (N=5 rats per condition at each time point); rats in the 70 day cohort were injected with 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) 50 mg/kg every 12 h over 2 days 6 weeks prior to sacrifice.

2.2 GK irradiation

Irradiation accuracy first was assessed with radiochromic film (GAFCHROMIC® MD-55, International Specialty Products) pierced by two straight pins orthogonal to the film plane (pin separation = 10.7 mm) and sandwiched within a cylindrical rodent phantom. The phantom and film were attached to the irradiation jig (Figure 1) and to a CT-compatible fiducial marker box and then imaged stereotactically using CT (see below). Using the images, the intersections of the pinholes with the film plane were targeted and irradiated for a maximum dose of 33 Gy (dose based on radiographic film sensitivity) using a 4 mm collimator. The center of each 4 mm dose distribution was located within 0.4 mm of the pinhole target (Figure 1C).

Rats were anesthetized using ketamine and xylazine (80 and 4 mg/kg body weight) and secured in a custom holding jig (Figure 1B), which was fixed to a Model G (Elekta AB) GK head frame and a fiducial marker box that provided marks for the reference coordinate system. The frame assembly and rat brain were imaged with a helical CT scanner; all animals, irradiated and sham irradiated, underwent anesthesia and CT imaging. For each irradiated rat, CT images were sent to the treatment planning workstation and an individual GK treatment plan was developed based on its unique CT data set. With the use of the CT images and stereotactic rat brain atlas [31], x, y, and z coordinates of the target region were calculated to align the animal for irradiation of the left dorsal hippocampus. The dose was targeted to the dorsal portion of the left hippocampus, centered 5.5 mm rostral to the coronal image where the external auditory meatus was observed bilaterally, 4 mm ventral to the dorsal surface of the brain, and 3.5–4 mm (depending on head rotation) lateral to the midline. A 4 mm collimator helmet was used and the dose distribution shaped with a custom plug pattern to reduce the dose contribution from lateral sources and elongate the dose

distribution in the anterior-posterior dimension (30% isodose lines and higher; Figure 2). This technique greatly minimized dose to the contralateral hemisphere. GK irradiation for experimental animals was at a nominal 2.2 Gy/min dose rate. Rats received either a single 10 Gy maximum dose or sham irradiation and then were returned to the home cage.

2.3 Tissue collection and immunolabeling

Animals were euthanized in matched pairs by sodium pentobarbital overdose (150 mg/kg) and perfusion fixed with 4% paraformaldehyde (PF). The brain was postfixed (4% PF, 4° C, 24 hours), cryoprotected, and cryosectioned. Coronal 40 µm sections were collected into cryoprotective solution and stored at −20 °C until processed for immunohistochemistry (IHC) and IF. The DNA stain Sytox Green (Molecular Probes/Invitrogen) was used to label sections from the 6 h time point to assess condensed and pyknotic nuclei, morphological indicators of apoptosis. An additional section from this group was stained with mouse monoclonal antibody against phosphorylated histone H2A.X (anti-gamma-H2A.X, clone JBW301, Millipore), a marker of complex DNA damage, including double strand breaks [30]. For other antibodies, a 1-in-12 series of sections representing the anterior-posterior extent of the dorsal hippocampus (from approximately bregma − 1.5 to bregma − 4.5 [31]) was labeled. Sections from each animal were labeled by IHC using published protocols [32] [33]. Antibodies against the lysosomal protein CD68 (ED1 clone [34], AbD Serotec) and the rat RT1B MHC class II antigen (OX6 clone, AbD Serotec [35]) labeled activated macrophages/microglia. Rabbit anti-Iba1 (Wako) labeled all macrophages/microglia [36]. For analysis of BrdU and cell type specific markers, sections were labeled by triple IF using rat anti-BrdU (Accurate Scientific), mouse anti-NeuN (a marker of mature neurons, Millipore), and anti-Iba1 according to published methods [33].

2.4 Quantitative and statistical analyses

Analyses were performed blindly using coded sections. In sections labeled by IHC we counted immunolabeled cells exhaustively within the subgranular zone (SGZ) of the DG and the combined granule cell layer (GCL) and hilus using a modification of the optical dissector method [37] and the NeuroLucida system (Microbrightfield, Inc.). Labeled cells were identified as within the SGZ if they were within 25 µm of the border between the GCL and hilus. The length of the SGZ and the area of the GCL/hilus were measured using NeuroLucida. For each immunolabel in each animal, the cell counts and the linear or areal measurements were summed across all sections in the 1-in-12 series. Densities then were calculated as # cells/mm of SGZ or # cells/mm³ of GCL/hilus. Fluorescently labeled cells were analyzed in the SGZ and GCL/hilus using stacks of optical sections obtained using a Leica TCS SP2 confocal microscope (Leica Microsystems). All BrdU⁺ cells that expressed either NeuN or Iba1 were examined in each of two sections from each animal.

For each dependent variable, the mean density of labeled cells was calculated for each group and compared by Analysis of Variance (ANOVA) using Sigmastat software (SYSTAT Software). Given the need to compare the targeted and contralateral hemispheres in GK irradiated rats, the hemisphere (rather than the animal) was used as the unit of variance for statistical analyses. For each dependent variable examined in the rats in Group 2, two-way ANOVA tested for effects of condition (GK targeted or contralateral hippocampus or sham hippocampus), time after treatment (1, 7, or 70 days), and interactions between the two variables. For analysis of neurogenesis in Group 2, one-way ANOVA tested for effects of condition. Post hoc comparisons (Holm-Sidak test) were completed in analyses for which ANOVA revealed significant differences among groups. The threshold of significance was $p \leq 0.05$.

3. Results

3.1 Focal radiation was accurately targeted to the left hemisphere

Quantitative analysis using dose-volume histograms (DVHs, Figure 2F–G) showed the high dose region was confined to the targeted hemisphere and hippocampal regions. The maximum dose (10 Gy) occurred at a point within the lateral aspect of the left hippocampus with an irradiated volume that was close to zero, as expected for the 4 mm GK dose distribution. The targeted, left hippocampal region received approximately 50 to 60 times greater average dose than the contralateral, right hippocampus. At least 95% of the left hippocampal region received > 3 Gy and the average dose was 6 Gy, whereas in the contralateral, right hemisphere > 90% of the hippocampal region received < 0.2 Gy and the average dose was < 0.1 Gy (range 0.0 to 0.3 Gy).

3.2 Acute cell death was restricted to the irradiated hippocampus

As measures of acute effects of unilateral GK irradiation, we assessed two rapid and sensitive markers of radiation-induced damage: DNA double-strand breaks and apoptosis in the SGZ of the DG [11] [30]. At 6 h after GK treatment (Group 1), qualitative analysis of gamma-H2A.X labeling, a marker of DNA damage, demonstrated increased immunolabeling in the irradiated hippocampus (Figure 3C). Labeling in the contralateral hippocampus (Figure 3B) appeared slightly elevated above sham controls (Figure 3A) but was much lower than in the targeted hippocampus. The same pattern of gamma-H2A.X labeling was evident in three GK irradiated rats. To assess whether there was sufficient DNA damage in either hemisphere to induce cell death, we counted apoptotic cells in the SGZ of the targeted and contralateral hemispheres of three GK irradiated rats and in four hemispheres from sham, control rats (Figure 3D). At 6 h, pyknotic nuclei (Figure 3E) were increased 10-fold in the targeted SGZ, but the number of pyknotic nuclei in the contralateral SGZ of GK irradiated rats did not differ from that in the SGZ of non-irradiated, sham rats (Figure 3F).

3.3 Inflammatory changes were restricted to the irradiated hippocampus

Rats in Group 2 were treated in the same manner as those in Group 1 but survived for 1, 7 or 70 days after treatment. The density of microglia labeled with the ED1 antibody, which recognizes a lysosomal antigen that is upregulated in activated microglia, was assessed in the DG SGZ and in the combined GCL/hilus (Figure 4 A–C). The density of ED1⁺ cells was affected by GK-treatment and by the time after irradiation, with a significant interaction between the factors (Table 1). Post hoc tests revealed no change at 1 day post-irradiation, but the density of ED1⁺ cells was increased in the targeted hippocampus (compared to the contralateral and sham hippocampus) at 7 days post-irradiation ($p < 0.01$ for both SGZ and GCL/hilus; Figure 5A and B) and remained significantly higher at 70 days post-irradiation ($p < 0.01$ for both regions). In the contralateral hemispheres of GK rats and in sham controls, the density of ED1⁺ cells appeared to be greater in the 70 day post-irradiation group than in the 1 and 7 day post-irradiation groups (Figure 5A, B), presumably reflecting an aging-related increase in microglial activation. The density of ED1⁺ cells in the contralateral hippocampus of GK rats never differed from that in sham irradiated controls.

The density of cells labeled for major histocompatibility class II antigen (MHC-II), a cell surface antigen that is increased in activated microglia, also increased in the targeted hippocampus following GK irradiation. Within both the SGZ and the GCL/hilus (Figure 4 D–F), the density of MHC-II⁺ cells was affected by radiation and by time following irradiation, with a significant interaction between the two factors (Table 1). Post hoc tests revealed an increase in the density of MHC-II⁺ microglia in the targeted hippocampus compared to the contralateral hippocampus and sham controls at 1 and 70, but not at 7, days

after GK-irradiation (Figure 5C and D; 1 d and 70 d $p < 0.01$, but 7 d $p > 0.05$). Thus, MHC-II expression was rapidly and specifically up-regulated in the irradiated hippocampus, decreased to basal levels by 7 days, and then showed a second elevation by 70 days after GK irradiation. There was no significant difference between sham controls and the contralateral hippocampus of GK animals at any time point, and little indication that the density of MHC-II⁺ cells in the contralateral hippocampus of GK animals or in sham animals changed across the survival times.

Total microglial density (density of Iba1⁺ cells) in the SGZ was affected by GK-irradiation and by time after irradiation, with a significant interaction (Table 1; Figure 6). At 1 day post-GK, the microglial density was greater in the targeted SGZ than in shams ($p < 0.05$, the difference between the targeted and contralateral hippocampus in GK rats did not quite reach significance at that time point, $p = 0.056$). In contrast to the acute increase, the density of microglia in the targeted hippocampus of GK rats was decreased at 7 and 70 days post-GK ($p < 0.01$ versus contralateral or sham controls). Microglial density remained constant across the three time points in the contralateral hippocampus of GK rats and in sham controls and never differed between those two conditions.

3.4 Changes in neurogenesis were restricted to the irradiated hemisphere

GK irradiation altered generation of neurons and microglia, as assessed by phenotypic analysis using triple immunofluorescent (IF) labeling for BrdU, the mature neuronal marker NeuN, and Iba1 (Figure 7; 70 day survival cohort, BrdU incorporation 6 weeks prior to analysis). In sham controls and in the contralateral hemisphere of GK rats, neurogenesis was substantially greater than microgliogenesis; there were six times as many BrdU⁺/NeuN⁺ cells as BrdU⁺/Iba1⁺ cells within the cohort of newborn cells in the GCL and SGZ (Figure 8). In the irradiated hippocampus, however, BrdU⁺/NeuN⁺ cells were reduced and BrdU⁺/Iba1⁺ cells were increased (Figure 8), indicating that neurogenesis was decreased and microglial turnover increased in the weeks following GK irradiation.

4. Discussion

We targeted a 6 Gy average (10 Gy max) dose of γ -radiation to the adult rodent hippocampus in one hemisphere and compared the neurobiological response in the target region and in the contralateral hippocampus to sham animals. This approach delivered to one hemisphere a dose range that has been tested previously in studies of WBI (see Introduction). The dose distribution was shaped elliptically to minimize irradiation of the contralateral hemisphere, so that we could test for out-of-field responses. GK irradiation induced several short- and longer term responses in the targeted hippocampus: i) acute apoptosis and a sustained decrease in neurogenesis in the SGZ of the DG, and ii) acute and chronic increases in microglial activation and changes in microglial population density. Overall, the response of the targeted hippocampus to focal irradiation was comparable to the response to WBI in a similar dose range [16] [32] [38] and all changes were restricted to the targeted hippocampus.

4.1 Cell death and effects on neurogenesis

In the contralateral hemisphere of GK-treated rats, there appeared to be a slight increase in DNA double-strand breaks, but any damage in that hemisphere was insufficient to induce detectable death of hippocampal progenitor cells or changes in neurogenesis. Previous analyses following WBI demonstrated changes in proliferation and neurogenesis in the SGZ in response to as little as 0.5 Gy and a steep dose response between 0 and 2 Gy [11] [12] [38]. In this study, CT imaging delivered approximately 0.01–0.02 Gy to the entire brain and GK irradiation delivered to the contralateral hippocampus an average of 0.1 Gy and a

maximum of ~ 0.3 Gy (at the medial margin of the DG). The present observation that irradiation up to 0.3 Gy in the contralateral DG of GK-treated rats produced no detectable cell death or loss of neurogenesis, along with previous demonstrations of progenitor cell death and neurogenic changes in response to 0.5 Gy and greater WBI [12], indicates that 0.5 Gy likely is close to the threshold for cell death and changes in neurogenesis. Given that shell or off-target regions in patients treated with SRS, 3D conformal radiotherapy, or IMRT can be exposed to doses at or above 0.5 Gy, protecting progenitor cell populations in the hippocampus and in the subventricular zone [1] [2] [3] [4] [39] during clinical RT may remain challenging. Additional experiments to better establish in vivo the radiation fraction dose-response for death of progenitor cells, changes in neurogenesis, and other functional changes in neurogenic regions will be important.

4.2 Microglial activation

Based on two markers of “activated” microglia, the radiation-induced microglial response (like that for acute cell death and effects on neurogenesis) was restricted to the targeted hippocampus. The temporal and spatial patterns of microglial changes revealed here demonstrate complexity in the microglial responses to pro-inflammatory stimuli and some independence of the regulation of different responses, adding to the growing recognition that microglial responses range across a multi-dimensional continuum of functional and phenotypic changes, rather than representing a switch between “resting” and “activated” states [40] [41].

Within the irradiated hemisphere of GK rats, the extent and time course of the inflammatory response within the SGZ and in the GCL/hilus as a whole depended on which marker of microglial activation was assessed (Figure 5). Expression of the lysosomal CD68 antigen (demonstrated by ED1 labeling) did not differ from control levels 1 day after GK irradiation but increased several fold by 7 and 70 days. This temporal pattern is consistent with our previous analysis of the response to 10 Gy WBI in rats of the same age [32]. In contrast to CD68 expression, MHC-II expression increased significantly by 1 day post-irradiation, returned to the control level at 7 days, and then increased again by 70 days post-irradiation. Thus, i) the initial response of microglia to irradiation (and/or the radiation-induced death of progenitor cells) involved increased expression of molecules related to antigen presentation (MHC II) but no detectable concurrent change in expression of the lysosomal marker CD68, and ii) subsequent changes in the two markers also occurred independently. Similarly, CD68 expression appeared to increase with age in sham rats and in the contralateral hemispheres of GK rats (comparing the 70 day survival group versus the 1 and 7 day survival groups), whereas MHC II expression did not. Clearly the phenotypic markers associated with different functional changes in “activated” microglia are regulated, to some extent, independently.

There were regional, as well as temporal, differences in the components of the microglial response. The timing of microglial responses to focal irradiation was similar in the SGZ and GCL/Hilus, but the magnitude of the responses varied between these regions of the DG. At 1 day after GK irradiation, MHC-II⁺ cells outnumbered ED1⁺ cells approximately ten-fold in the GCL/hilus but only about five-fold in the SGZ. At 70 days after GK irradiation, MHC-II⁺ cells outnumbered ED1⁺ cells approximately two-fold in the GCL/hilus, but the densities of ED1⁺ and MHC-II⁺ cells were similar in the SGZ. Therefore, following irradiation, MHC-II⁺ cells outnumbered ED1⁺ cells to a greater extent outside of the proliferative zone (in the GCL/hilus) than within the proliferative zone (in the SGZ). Such evidence that microglial responses to an experimental challenge and to aging are regionally heterogeneous emphasizes the importance of assessing the effects of focal irradiation and comparing homologous regions of irradiated and un-irradiated hemispheres in individual subjects.

In principle, the differential responses of different microglial markers following GK treatment could represent combined responses of intrinsic microglia and infiltrating myeloid cells (macrophages), since the microglial markers used here (as in most studies) do not differentiate intrinsic microglia from macrophages. Significantly, however, previous analysis of chimeric animals obtained by parabiosis demonstrated directly that brain irradiation at 10 Gy was not sufficient to result in migration of macrophage precursors from the periphery into the CNS in young adult rodents [42]. Thus, the radiation-induced inflammatory changes seen here, and the temporal and spatial differences in cell responses to irradiation, likely reflect complex responses of the population of intrinsic microglia.

4.3 Conclusion

Accumulating experimental evidence indicates that the responses of neural cells to relatively low dose irradiation contribute to radiation-induced cognitive dysfunction [10] [13] [14] [15] [43] [44]. Significantly, cognitive deficits occur in experimental models in the absence of detectable radiation necrosis or other gross pathological changes [23] [45] [46] and, in the clinic, radiation-induced cognitive dysfunction may develop before or in the absence of detectable changes in imaging and pathological assessments [47] [48]. Thus, the critical mechanisms of radiation-induced neural dysfunction appear to involve complex changes in intercellular signaling and cell function that may be relatively subtle. Clearly a variety of in vivo and in vitro strategies are required to develop and test mechanistic models [49]. We developed an approach here to better understand how components of radiotherapy contribute to neural dysfunction and determined that focally restricted irradiation at a dose that is delivered to peri-target regions during clinical SRS induces changes in neurogenesis and inflammation similar to those seen following WBI. No such changes were evident in adjacent tissue that received a dose of 0.3 Gy or less. Additional assessment of molecular changes within targeted and out-of-target tissues, as well as more extensive evaluation of inflammatory changes, should facilitate development and testing of more effective therapeutic strategies to preserve function following radiation therapy.

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Figure 1. GK irradiation of young adult rats

A young adult rat is shown positioned in the modified model G head frame (A) and then attached to a 4 mm collimator helmet (B). Radiographic film irradiated in a cylindrical phantom shows GK irradiation occurs with high precision and accuracy (C); the center of each dose distribution was located within 0.4 mm of the pinhole used to target the dose.

Figure 2. Dose planning and DVH analysis

Isodose lines representing 90%, 70%, 50%, 30% and 10% of the maximum 10 Gy dose are shown on CT images of the rat head in the coronal (A, posterior is upper left, anterior is lower right), sagittal (B), and axial planes (C). To better relate the dose distribution to neuroanatomical landmarks, isodose estimates of 90%, 50%, and 10% of maximum were plotted on coronal and sagittal sections of a rat brain atlas [44] (D, E); the contralateral hippocampus is indicated in black. Dose volume histograms (F) were evaluated for the targeted (squares) and contralateral (circles) hemispheres (open symbols and left ordinate) and hippocampus (closed symbols and right ordinate). The dose distribution in each

hippocampus is replotted (G) on an expanded dose scale and as a percentage of the total hippocampal volume.

Figure 3. Acute effects of GK irradiation

Gamma-H2A.X labeling at 6 h after GK SRS or sham irradiation is illustrated in the right DG of a sham irradiated control (A), the right, contralateral hippocampus of a GK irradiated rat (B), and the left, targeted hemisphere of a GK irradiated rat (C, higher magnification in D). Dying cells were identified using Sytox Green staining of DNA, which revealed pyknotic nuclei in the targeted DG of GK irradiated rats (E, inset shows the cluster of dying cells indicated by the arrow). Cell death was quantified by counting pyknotic nuclei in the SGZ (F, values shown are mean+sem). Scale bar = 250 μ m (A–C), 25 μ m (D, E).

Figure 4. ED1 and MHC-II immunolabeling

Activated microglia were demonstrated by immunolabeling with antibodies to ED1 (shown at 7 days after GK or sham treatment, A–C) and MHC-II (shown at 70 days after GK or sham treatment, D–F). In these combined epifluorescent and brightfield images, DAPI labeling reveals the cell-dense granule cell layer bounding the hilus of the DG, while ED1⁺ or MHC-II⁺ cells appear black. Labeling is shown for the DG of a sham irradiated control rat (A, D), the contralateral DG of GK irradiated rat (B, E), and the targeted DG of a GK irradiated rat (C, F). Insets in C and F show details of ED1 and MHC-II labeling. Scale bar = 125 μ m, 20 μ m (insets).

Figure 5. Quantitative analysis of ED1⁺ and MHC-II⁺ cells in the SGZ

Mean (+sem) densities of ED1⁺ cells (A and B) and MHC-II⁺ cells (C and D) in the SGZ (A, C) and in the GCL/hilus (B, D) are shown for sham irradiated rats (open bars), contralateral hemispheres of GK irradiated rats (gray bars), and targeted hemispheres of GK irradiated rats (black bars) at 1, 7 and 70 days after sham or GK irradiation. * $p < 0.01$ vs sham and contralateral GK; # $p < 0.01$ vs GK targeted hemisphere at 1 or 70 days post-irradiation (two-way ANOVA followed by Holm-Sidak post-hoc tests).

Figure 6. Quantitative analysis of microglial density

The density of Iba1⁺ cells (mean±sem) is shown for the SGZ in unirradiated sham rats (open bars), the contralateral hemisphere of GK irradiated rats (gray bars), and targeted hemisphere of GK irradiated rats (black bars). * $p < 0.01$ vs sham or contralateral GK, # $p < 0.01$ vs 1 day post-irradiation (two-way ANOVA followed by Holm-Sidak post-hoc tests).

Figure 7. Phenotypic identification of newborn cells in the DG

Triple IF labeling is shown for BrdU (red), NeuN (blue) and Iba1 (green). Portions of the GCL and SGZ in the DG of the contralateral and targeted hemispheres of a GK irradiated rat are illustrated. In the contralateral hemisphere (A–D), there is a BrdU⁺/NeuN⁺ neuron (long arrows) that, as expected, lacks Iba1 labeling. Cell bodies of Iba1⁺ microglia (NeuN[−] and BrdU[−]) also can be identified (short arrows). In the targeted hemisphere (E–H), the field contains no BrdU⁺ neurons, but a BrdU⁺ microglial cell is evident in the SGZ (arrow). Scale bars = 18 μ m (A–D), 12 μ m (E–H).

Figure 8. Quantitative analysis of phenotypic differentiation of newborn cells in the DG

The percentages (mean+sem) of newborn cells in the GCL and SGZ that were identified as neurons (BrdU⁺/NeuN⁺), as microglia (BrdU⁺/Iba1⁺), or neither (BrdU⁺/NeuN⁻/Iba1⁻) are shown for unirradiated sham controls, the contralateral hippocampus of GK irradiated rats (GK-C), and the targeted hippocampus of GK irradiated rats (GK-IR). * $p \leq 0.01$ versus sham or GK-C, ** $p \leq 0.001$ versus sham or GK-C (one-way ANOVA followed by Holm-Sidak post-hoc tests).

Table 1

Results of primary statistical analysis.

		ED1	MHC-II	Iba1
SGZ	Condition	$F(2,36)=13.35, p<0.001$	$F(2,36)=33.90, p<0.001$	$F(2,36)=7.91, p<0.001$
	Time	$F(2,36)=13.41, p<0.001$	$F(2,36)=27.96, p<0.001$	$F(2,36)=11.41, p<0.001$
	Interaction	$F(4,36)=3.45, p<0.05$	$F(4,36)=12.98, p<0.001$	$F(4,36)=12.00, p<0.001$
GCL/hilus	Condition	$F(2,36)=18.65, p<0.001$	$F(2,36)=31.60, p<0.001$	NA
	Time	$F(2,36)=12.08, p<0.001$	$F(2,36)=32.15, p<0.001$	NA
	Interaction	$F(4,36)=4.34, p<0.01$	$F(4,36)=18.74, p<0.001$	NA

Results are shown for ANOVAs testing for effects of condition (GK targeted, GK contralateral, or sham), time after irradiation (1, 7 or 70 days), and interactions on inflammatory markers in young adult rats. Results from counts in the SGZ and GCL/hilus were analyzed separately.